Exhibit A

Biotechnological Aspects: Methods

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Inverted-repeat DNA: a new gene-silencing tool for seed lipid modification

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Abstract

Post-transcriptional gene silencing (PTGS) has been successfully used to modify seed lipids in oilseed crops like soybean, canola and sunflower. Conventionally, PTGS has been induced by transforming the plants with either antisense or co-suppression constructs targeted against key seed lipid biosynthesis genes. A major drawback of this approach has been the recovery of only a modest proportion of silenced individuals from large populations of transgenic plants. In this report we show that inverted-repeat DNA constructs containing an intron encoding RNA with a hairpin structure can induce PTGS with very high frequency.

Introduction

Plant breeders frequently seek to improve crops by down-regulating expression of genes encoding undesirable traits. They have done this traditionally by selecting within natural variation inherent in a species, often needing to use unadapted germplasm as the source of the required trait expression. This is a slow and costly process requiring several generations and considerable effort to construct elite lines with the desired phenotype. Alternatively, new variation for trait expression can often be generated through the use of chemical mutagens or ionizing radiation. In this case, large mutagenized populations have to be created and screened, and desirable selections may then have to undergo extensive backcrossing to remove random mutations not associated with the trait and deleterious to agronomic performance. A further limitation in using induced mutation approaches is that they cannot control gene expression in a tissue-specific manner if the genes involved have constitutive expression. For example, the first rapeseed mutants selected for high oleic acid content showed unfavourable characteristics like low vigour, disease susceptibility and low seed yield [1]. This was most likely a consequence of the mutations being in genes encoding both seed and vegetative forms of the Δ12-desaturase, whereas the down-regulation of just the seed-specific *FAD2* locus for this enzyme resulted in a large increase in oleic acid with little or no adverse effect on plant performance [2].

Post-transcriptional gene silencing (PTGS), a sequence-specific RNA degradation mechanism inherent in eukaryotes, has been successfully used to silence gene expression and produce desirable traits in crop plants. PTGS has been induced in plants either through the use of antisense or co-suppression constructs [3]. For example, fatty acid composition of oilseeds has been successfully altered through the silencing of the Δ9-desaturase [4] and Δ12-desaturase [2] genes. One major advantage of this approach is the ability to avoid any undesirable effects of global silencing of the target gene by confining the gene suppression to a specific tissue or organ through the use of appropriate tissue-specific promoters to drive the gene-silencing constructs. However, the relatively low frequency of PTGS achieved with antisense and co-suppression [5] requires that large populations of transgenic plants are produced in order to obtain an acceptable number of transgenic lines exhibiting sufficient degrees of target gene suppression. This can present a major limitation,

Key words: *Arabidopsis*, Δ12-desaturase; dsRNA.
Abbreviations used: PTGS, post-transcriptional gene silencing; ODP, oleic desaturation proportion.
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particularly in species that have low transformation and regeneration frequencies. In this and an accompanying article by Liu et al. [6] we describe the use of inverted-repeat DNA constructs recently described by Waterhouse et al. [7] to achieve very high efficiency silencing of desaturase genes in *Arabidopsis* and cotton.

Experimental

The isolation and characterization of the *Arabidopsis* Δ12-desaturase (at*FAD2*) gene sequence used in this study was described earlier [8]. The longest region of the gene used in the present study comprised a 5′-truncated cDNA fragment (1103 bp) of the at*FAD2* gene. Seed-specific gene-silencing constructs in *Arabidopsis* were driven by the Fp1 napin promoter. Inverted-repeat DNA gene-silencing constructs were made according to the principles described earlier [7] and transformed into the Columbia ecotype of *Arabidopsis thaliana* by *Agrobacterium tumefaciens* transformation. Fatty acids of bulk T_3 seed from T_1 primary transformants were analysed by GC. The magnitude of Δ12-desaturase gene silencing has been calculated as the reduction in the oleic desaturation proportion (ODP) parameter, which is an indicator of the Δ12-desaturase activity, derived by the formula

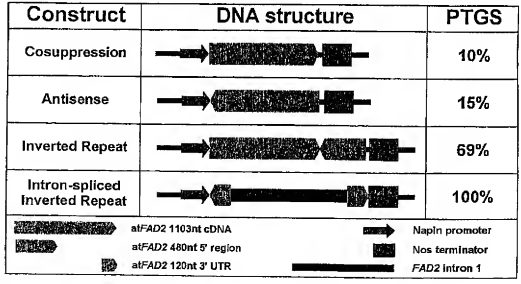
$$\text{ODP} = (\%\ 18{:}2 + \%\ 18{:}3)/(\%\ 18{:}1 + \%18{:}2 + \%\ 18{:}3)$$

Results and discussion

Homology-dependent gene silencing has been frequently shown to occur between transgenes and endogenous genes having high degrees of sequence homology. One form of homology-dependent gene silencing is PTGS, which is exemplified by silencing of homologous endogenous genes in response to the expression of co-suppression or antisense constructs [3]. Figure 1 summarizes the effect of expressing various silencing constructs targeted against the *Arabidopsis* Δ12-desaturase. A co-suppression-type construct based on the 5′-truncated cDNA fragment caused 10% of the 41 independent transgenic plants to show PTGS, as evidenced by a greater than 20% reduction in the ODP parameter in the seed lipids. Similarly, it was shown previously that an antisense construct using the full-length at*FAD2* gave ≈15% of transgenics (3/21) exhibiting PTGS [1]. We also

Efficiency of PTGS induction by different gene constructs

PTGS efficiency was measured as the proportion of transgenic plants showing a greater than 20% reduction in Δ12-desaturase activity when compared with the wild type.



produced an inverted-repeat silencing construct by placing the 480 bp 5′ region of the 5′-truncated cDNA fragment in antisense orientation at the 3′ end, effectively producing a 623 bp spacer region between the flanking inverted-repeat arms. When this construct was expressed in *Arabidopsis*, ≈ 69% of the transgenics (44/63) showed PTGS. Even more interestingly, when the spacer region in the inverted-repeat construct was replaced with the *atFAD2* intron 1, which should get spliced out, and the flanking inverted-repeat arms were reduced to just the 120 bp 3′ untranslated region, 100% (30/30) of the transgenics showed PTGS of Δ12-desaturase. Furthermore, both inverted-repeat DNA constructs were able to produce similar high degrees of PTGS in either the hemizygous or homozygous state. This was in contrast to the co-suppression or antisense constructs where higher degrees of PTGS were seen in homozygotes than in heterozygotes (results not shown). Also, in an accompanying article by Liu et al. [6], we show that the inverted-repeat DNA type of constructs were far superior in inducing PTGS in cotton than were conventional antisense constructs.

This intron-mediated enhancement of PTGS was shown to be linked to the intron being spliced out by the results reported in [9], where lower levels of PTGS were associated with a construct where the intron was included but was not able to be spliced out. We can advance at least two explanations to account for this intron-enhanced silencing efficiency. First, intron excision by the spliceosome might help align the complementary arms of the hairpin in a favourable environment for RNA hybridization, promoting the formation of an RNA duplex that has been shown to be the trigger for PTGS [3,7]. Secondly, splicing may increase the transient level of duplex RNA by altering the passage of the duplex from the nucleus, or by providing a smaller, less nuclease-sensitive, loop.

In conclusion, we have described here the design of gene-silencing constructs that induce a very high frequency of PTGS in plants. The use of small unique regions of genes, like the 3′ untranslated region coupled with a splicing intron, should be as useful to plant biology as RNA interference has become for the study of nematodes and *Drosophila* [10].

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Genetic modification of cotton seed oil using inverted-repeat gene-silencing techniques

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Abstract

Inverted-repeat-based gene constructs targeted against two key cotton seed-specific fatty acid desaturase genes, *ghSAD-1*, encoding stearoyl-acyl carrier protein Δ9-desaturase and *ghFAD2-1*, encoding microsomal ω-6 desaturase, were transformed into cotton. The expression of *ghSAD-1* and *ghFAD2-1* in the inverted-repeat orientation resulted in increased levels of stearic and oleic acids, respectively. Interestingly, the content of palmitic acid in both high-stearic and high-oleic lines was substantially reduced. These materials offer the promise of developing cotton seed oil

Key words: desaturase, fatty acid.
Abbreviations used: LDL, low-density lipoprotein; ACP, acyl carrier protein.
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